

## AMENDMENT TO GLP TEST PROTOCOL

Amendment No.: 2

Effective Date: 8/22/11

Sponsor: Albemarle Corporation  
Process Development Center  
PO Box 341  
Baton Rouge, LA 70801EXACT COPY  
INITIALS MS DATE 9-1-11Test Facility: ATS Labs  
1285 Corporate Center Drive, Suite 110  
Eagan, MN 55121

Protocol Title: AOAC Available Chlorine in Disinfectants

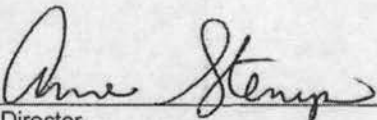
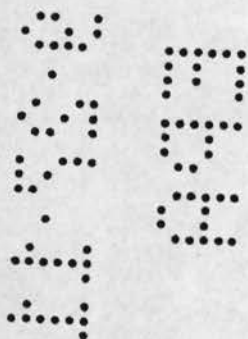
ATS Labs Protocol Number: ABM01052511.AVC.2

ATS Labs Project Number: A11810

## Modifications to Protocol:

Per Sponsor's request and due to the fact the Neutralization Confirmation Control performed for DBDMH Lot 100720 and 100706 at the 200 ppm concentration failed to support growth of the test organism, testing for DBDMH Lot 100720 and 100706 at the 200 ppm concentration is canceled prior to the generation of valid data.

Changes to the protocol are acceptable as noted.

  
Study Director8-23-11  
Date

AMENDMENT TO GLP TEST PROTOCOL

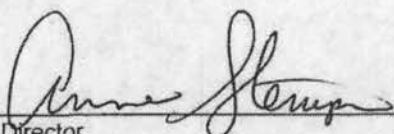
Amendment No.: 1  
Effective Date: 8/17/11  
Sponsor: Albemarle Corporation  
Process Development Center  
PO Box 341  
Baton Rouge, LA 70801  
Test Facility: ATS Labs  
1285 Corporate Center Drive, Suite 110  
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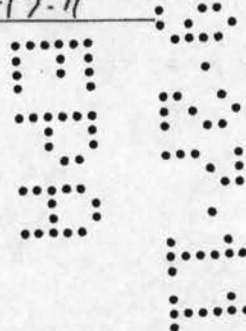
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INITIALS h DATE 9-1-11

Modifications to Protocol:

Due to the fact that the 50 ppm, 100 ppm and 200 ppm concentrations of the NaOCl Control Solutions did not demonstrate any visual growth, this protocol is amended to allow for the subculture of each individual tube used for the NaOCl Control solutions to the appropriate agar media.

Changes to the protocol are acceptable as noted.

  
Study Director

8-17-11  
Date 

(For Laboratory Use Only)  
ATS Labs Project # **A11810**

*LC 4/10/11*

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PROTOCOL

AOAC Available Chlorine in Disinfectants

Test Organism:

Listeria monocytogenes

ATCC 19117

PROTOCOL NUMBER

ABM01052511.AVC.2

*\*(test organism name should be in italics)*

*\* information added by the study director on 8-9-11*

PREPARED FOR

Albemarle Corporation  
Process Development Center  
PO Box 341  
Baton Rouge, LA 70801

PERFORMING LABORATORY

ATS Labs  
1285 Corporate Center Drive, Suite 110  
Eagan, MN 55121

PREPARED BY

Anne Stemper, B.S.  
Research Scientist I

DATE

May 25, 2011

PROPRIETARY INFORMATION

THIS DOCUMENT IS THE PROPERTY OF AND CONTAINS PROPRIETARY INFORMATION OF ATS LABS. NEITHER THIS DOCUMENT, NOR INFORMATION CONTAINED HEREIN IS TO BE REPRODUCED OR DISCLOSED TO OTHERS, IN WHOLE OR IN PART, NOR USED FOR ANY PURPOSE OTHER THAN THE PERFORMANCE OF THIS WORK ON BEHALF OF THE SPONSOR, WITHOUT PRIOR WRITTEN PERMISSION OF ATS LABS.



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### AOAC Available Chlorine in Disinfectants

**SPONSOR:** Albemarle Corporation  
Process Development Center  
Baton Rouge, LA 70801

**TEST FACILITY:** ATS Labs  
1285 Corporate Center Drive, Suite 110  
Eagan, MN 55121

#### **PURPOSE**

The purpose of this study is to determine the available chlorine germicidal equivalent concentrations with products offered for use as sanitizing rinses for previously cleaned nonporous surfaces following the AOAC Chlorine (Available) in Disinfectants Method. This method is in compliance with the U. S. Environmental Protection Agency (EPA).

#### **TEST SUBSTANCE CHARACTERIZATION:**

Test substance characterization as to content, stability, etc., (40 CFR, Part 160, Subpart F [160.105]) is the responsibility of the Sponsor. The test substance shall be characterized by the Sponsor prior to the experimental start date of this study. Pertinent information, which may affect the outcome of this study, shall be communicated in writing to the Study Director upon sample submission to ATS Labs.

#### **SCHEDULING AND DISCLAIMER OF WARRANTY**

Experimental start dates are generally scheduled on a first-come/first-serve basis once ATS Labs receives the Sponsor approved/completed protocol, signed fee schedule and corresponding test substance(s). Based on all required materials being received at this time, the proposed experimental start date is June 2, 2011. Verbal results may be given upon completion of the study with a written report to follow on the proposed completion date of June 28, 2011. To expedite scheduling, please be sure all required paperwork and test substance documentation is complete/accurate upon arrival at ATS Labs.

If a test must be repeated, or a portion of it, due to failure by ATS Labs to adhere to specified procedures, it will be repeated free of charge. If a test must be repeated, or a portion of it, due to failure of internal controls, it will be repeated free of charge. "Methods Development" fees shall be assessed, however, if the test substance and/or test system require modifications due to complexity and difficulty of testing.

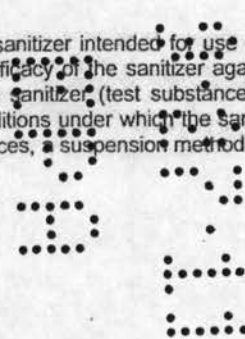
If the Sponsor requests a repeat test, they will be charged for an additional test.

Neither the name of ATS Labs nor any of its employees are to be used in advertising or other promotion without written consent from ATS Labs.

The Sponsor is responsible for any rejection of the final report by the regulatory agencies concerning report format, pagination, etc. To prevent rejection, Sponsor should carefully review the ATS Labs final report and notify ATS Labs of any perceived deficiencies in these areas before submission of the report to the regulatory agency. ATS Labs will make reasonable changes deemed necessary by the Sponsor, without altering the technical data.

#### **JUSTIFICATION FOR SELECTION OF THE TEST SYSTEM**

Regulatory agencies require that a specific bacterial claim for a halogen based sanitizer intended for use on food contact surfaces be supported by appropriate scientific data demonstrating the efficacy of the sanitizer against the claimed bacteria. This is accomplished by treating the target bacteria with the sanitizer (test substance) under conditions which simulate as closely as possible, in the laboratory, the actual conditions under which the sanitizer is designed to be used. For sanitizer products intended for use on food contact surfaces, a suspension method is used in the generation of the supporting data.



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**TEST PRINCIPLE**

A suspension of bacterial cells is exposed to the sanitizer and control NaOCl solutions of known concentrations for a specified exposure time. After exposure, an aliquot of the exposed suspension is transferred to tubes containing neutralizing subculture media, and the process is repeated. The subculture tubes are then incubated and assayed for survivors. Appropriate sterility, culture purity, viability and neutralization controls are performed. The current version of Standard Operating Procedure CGT-4020 reflects the methods which shall be used in this study.

**TEST METHOD**

Test Organism	ATCC #	Growth Medium	Incubation Parameters
<i>Listeria monocytogenes</i>	19117	Brain Heart Infusion broth	35-37°C, aerobic

The test organism to be used in this study was obtained from the American Type Culture Collection (ATCC), Manassas, VA.

**Preparation of Test Organism**

The growth medium is inoculated using a stock culture of test organism. A minimum of three transfers will be performed on consecutive days prior to use in testing procedures.

A 48-54 hour broth culture incubated at 35-37°C will be prepared. Vortex mix for 3-4 seconds and then let stand  $\geq 10$  minutes prior to use.

An organic soil load may be added to the test culture per Sponsor's request.

**Preparation of Test Substance**

The test substance shall be prepared according to the directions for intended use of the product. If the test substance is to be diluted, it shall be used within 3 hours of preparation. Transfer 10 mL of the test substance to sterile tubes. Place the tubes in a  $20 \pm 1^\circ\text{C}$  waterbath and equilibrate for  $\geq 10$  minutes. Other temperatures may be used as specified by the sponsor.

**NaOCl Preparation**

NaOCl Control solutions are prepared containing 200, 100, and 50 ppm available chlorine. The 200 ppm solution is titrated and must be  $200 \pm 10$  ppm. From the 200 ppm solution, the 100 and 50 ppm concentrations will be made. Transfer 10 mL of each sample to sterile tubes. Place tubes in a  $20 \pm 1^\circ\text{C}$  waterbath and equilibrate for  $\geq 10$  minutes. Other temperatures may be used as specified by the sponsor.

**Exposure Conditions**

A 50  $\mu\text{L}$  aliquot of test culture is added to each of the Test Substance/control NaOCl solutions and a calibrated timer is started. Mix the inoculated substance and return the tube to the water bath. One minute after inoculation, transfer 10  $\mu\text{L}$  of the inoculated suspension to 10 mL of appropriate neutralizing subculture broth. Thirty seconds later, the test/control material is reinoculated with 50  $\mu\text{L}$  of test culture as before. One minute after the second inoculation, transfer 10  $\mu\text{L}$  of the inoculated suspension to a second tube containing 10 mL of appropriate neutralizing subculture broth. This inoculation/subculture routine is repeated until a total of 10 replicate subcultures for each Test Substance/NaOCl Control concentration have been performed.

**Incubation and Observation**

All subcultures and controls are incubated for  $48 \pm 4$  hours at 35-37°C (or other appropriate time/temperatures).

Following incubation, the subcultures will be visually examined for growth. If necessary, the subcultures may be placed at  $2-8^\circ\text{C}$  for up to three days prior to examination.

Minimally, the first test tube showing growth in each test run will be subcultured, stained and/or biochemically assayed to confirm or rule out the presence of the test organism.

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#### STUDY CONTROLS

##### **Purity Control**

A "streak plate for isolation" will be performed on the test organism culture(s) and following incubation examined in order to confirm the presence of a pure culture. The acceptance criterion for this study control is a pure culture demonstrating colony morphology typical of the test organism.

##### **Organic Soil Sterility Control**

The serum used for soil load will be cultured, incubated, and visually examined for growth. The acceptance criterion for this study control is lack of growth.

##### **Neutralizing Subculture Medium Sterility Control**

A representative sample of uninoculated neutralizing subculture medium will be incubated and visually examined for growth. The acceptance criterion for this study control is lack of growth.

##### **Viability Control**

A 50  $\mu$ L aliquot of each test organism suspension will be transferred to a tube of subculture medium. The subculture medium containing the aliquot will be incubated and visually examined for growth. The acceptance criterion for this study control is growth.

##### **Neutralization Confirmation**

When the test product subculture tubes in a given set demonstrate no growth following incubation, they will be challenged with low levels of the organism. Following incubation, at least one subculture tube (per test run) will be inoculated with  $\leq 100$  CFU of organism. The actual CFU added back will be enumerated by inoculating duplicate agar plates as was performed for the tubes. The tubes and plates will be incubated for  $48 \pm 4$  hours at  $35-37^{\circ}\text{C}$ . Following incubation the neutralization confirmation tubes will be examined for growth. The agar plates will be counted to verify the inoculum. This control may be performed with multiple replicates using different dilutions of the test organism. The acceptance criterion for this study control is growth.

##### **Initial Suspension**

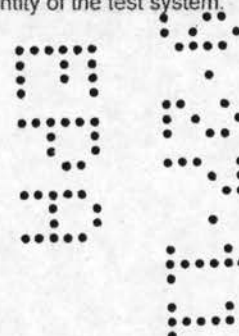
The initial suspension(s) will be serially diluted and plated using standard microbiological techniques. Following incubation at  $35-37^{\circ}\text{C}$  for  $48 \pm 4$  hours, the organism plates will be observed to enumerate the concentration of the test organism present at the time of testing. The acceptance criterion for this study control is a minimum of  $1.0 \times 10^6$  CFU/mL.

#### PROCEDURE FOR IDENTIFICATION OF THE TEST SYSTEM

ATS Labs maintains Standard Operating Procedures (SOPs) relative to efficacy testing studies. Efficacy testing is performed in strict adherence to these SOPs which have been constructed to cover all aspects of the work including, but not limited to, receipt, log-in, and tracking of biological reagents including bacterial strains for purposes of identification, receipt and use of chemical reagents. These procedures are designed to document each step of efficacy testing studies. Appropriate references to medium batch number, etc. are documented in the raw data collected during the course of each study.

Additionally, each efficacy test is assigned a unique Project Number when the protocol for the study is initiated by the Study Director. This number is used for identification of the test subculture tubes, etc. during the course of the test. Test subculture tubes are also labeled with reference to the test organism, experimental start date, and test product. Microscopic and macroscopic evaluations of positive subcultures are performed in order to confirm the identity of the test organism. These measures are designed to document the identity of the test system.

#### METHOD FOR CONTROL OF BIAS: N/A



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#### STUDY ACCEPTANCE CRITERIA:

##### **Test Substance Performance Criteria**

The activity equivalence of the germicide is determined as compared to the 200, 100, 50 ppm available chlorine in the NaOCl standard controls.

##### **Control Acceptance Criteria**

The study controls must perform according to the criteria detailed in the study controls description section. If any of the control acceptance criteria are not met, the test may be repeated under the current protocol number.

#### REPORT

The report will include, but not be limited to, identification of the sample, date received, initiation and completion dates, identification of the bacterial strains used, description of media and reagents, description of the methods employed, tabulated results and conclusion as it relates to the purpose of the test, and all other items required by 40 CFR Part 160.185.

#### PROTOCOL CHANGES

If it becomes necessary to make changes in the approved protocol, the revision and reasons for changes will be documented, reported to the Sponsor and will become a part of the permanent file for that study. Similarly, the Sponsor will be notified as soon as possible whenever an event occurs that may have an effect on the validity of the study.

Standard operating procedures used in this study will be the correct effective revision at the time of the work. Any minor changes to SOPs (for this study) or methods used will be documented in the raw data and approved by the Study Director.

#### TEST SUBSTANCE RETENTION

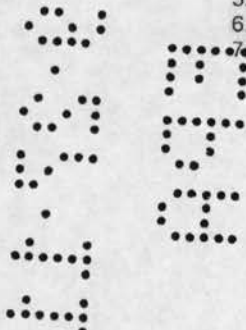
*It is the responsibility of the Sponsor to retain a sample of the test substance. All unused test substance will be discarded following study completion unless otherwise indicated by Sponsor.*

#### RECORD RETENTION

##### **Study Specific Documents**

All of the original raw data developed exclusively for this study shall be archived at ATS Labs. These original data include, but are not limited to, the following:

1. All handwritten raw data for control and test substances including, but not limited to notebooks, data forms and calculations.
2. Any protocol amendments/deviation notifications.
3. All measured data used in formulating the final report.
4. Memoranda, specifications, and other study specific correspondence relating to interpretation and evaluation of data, other than those documents contained in the final study report.
5. Original signed protocol.
6. Certified copy of final study report.
7. Study-specific SOP deviations made during the study.



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### Facility Specific Documents

The following records shall also be archived at ATS Labs. These documents include, but are not limited to, the following:

1. SOPs which pertain to the study conducted.
2. Non study-specific SOP deviations made during the course of this study which may affect the results obtained during this study.
3. Methods which were used or referenced in the study conducted.
4. QA reports for each QA inspection with comments.
5. Facility Records: Temperature Logs (ambient, incubator, etc.), Instrument Logs, Calibration and Maintenance Records.
6. Current curriculum vitae, training records, and job descriptions for all personnel involved in the study.

### REFERENCES

1. Official Methods of Analysis of the AOAC, Sixteenth Edition, 2005. Chapter 6 – Disinfectants, 955.16 Chlorine (Available) in Disinfectants, Germicidal Equivalent Concentration.
2. Association of Official Analytical Chemists (AOAC), 2005. Germicidal and Detergent Sanitizing Action of Disinfectants Method 960.09 [Preparation of Synthetic Hard Water].
3. U.S. Environmental Protection Agency, Registration Division, Office of Pesticide Programs, 1982. Subseries 91A: Public Health Uses. In Pesticide Assessment Guidelines – Subdivision G (Product Performance).
4. EPA DIS/TSS-4 Efficacy Data Requirements. Sanitizing Rinses (For Previously Cleaned Food Contact Surfaces). January 30, 1979.
5. EPA DIS/TSS-2 Efficacy Data Requirements, Supplemental Recommendations. January 25, 1979.

### DATA ANALYSIS

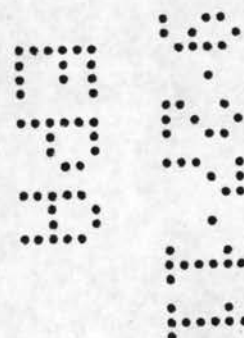
#### Calculations

The initial suspension plates will be enumerated as follows:

$$\text{CFU/mL} = \frac{(\text{Average CFU}) \times (\text{Dilution Factor})}{(\text{Volume plated in mL})}$$

#### Statistical Analysis

None used.



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### STUDY INFORMATION

(All sections must be completed prior to submitting protocol)

Test Substance (Name & Batch Numbers, including ≥60 day old batch - exactly as it should appear on final report):

DBDMH Lot 100700 & 100706

Specify ≥60 day old batch (if applicable): both samples are ≥ 60 days

Expiration Date:

7/2013

old \*

#### Product Description:

- ☐ Quaternary ammonia  
☐ Iodophor  
☐ Sodium hypochlorite

- ☐ Peracetic acid  
☐ Peroxide

☒ Other: Bromine based oxidizer

Test Substance Active Concentration (upon submission to ATS Labs):

Needs to be titrated

Neutralization/Subculture Broth:

- ☒ AOAC Fluid Thioglycollate Broth with 0.5% Sodium Thiosulfate  
☐ ATS Labs' Discretion. By checking, the Sponsor authorizes ATS Labs, at their discretion, to perform neutralization confirmation assays at the Sponsor's expense prior to testing to determine the most appropriate neutralizer. (See Fee Schedule).

#### Storage Conditions:

- ☒ Room Temperature  
☐ 2-8°C  
☐ Other:

#### Hazards:

- ☐ None known: Use Standard Precautions  
☒ Material Safety Data Sheet, Attached for each product  
☐ As Follows:

#### Product Preparation

- ☐ No dilution required, Use as received (RTU)  
☐ \*Dilution(s) to be tested:

defined as \_\_\_\_\_ \* \_\_\_\_\_  
(example: 1 oz/gallon) (amount of test substance) (amount of diluent)

- ☐ Deionized Water (Filter or Autoclave Sterilized)  
☐ Tap Water (Filter or Autoclave Sterilized)  
☐ AOAC Synthetic Hard Water: \_\_\_\_\_ PPM  
☒ Other: Chlorine demand free sterile water

\*Note: An equivalent dilution may be made unless otherwise requested by the Sponsor.

Test Organism:

☒ *Listeria monocytogenes* (ATCC 19117)

Exposure Temperature: 20±1°C

Organic Soil Load:

- ☐ Minimum 5% Organic Soil Load (Fetal Bovine Serum)  
☒ No Organic Soil Load Required  
☐ Other:

Test Substance mixing instructions:

- ☐ Test substance is viscous or may create foam during mixing and should be mixed by hand during testing.  
☒ Not applicable (test substance may be vortex mixed during testing)

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TEST SUBSTANCE SHIPMENT STATUS

- ☐ Has been used in one or more previous studies at ATS Labs.  
☐ Has been shipped to ATS Labs (but has not been used in a previous study).  
Date shipped to ATS Labs: \_\_\_\_\_ Sent via overnight delivery? ☐ Yes ☐ No  
☒ Will be shipped to ATS Labs.  
Date of expected receipt at ATS Labs: 7/29/11  
☐ Sender (if other than Sponsor): \_\_\_\_\_

COMPLIANCE

Study to be performed under EPA Good Laboratory Practice regulations (40 CFR Part 160) and in accordance to standard operating procedures.

- ☒ Yes  
☐ No (Non-GLP Study)

PROTOCOL MODIFICATIONS

- ☐ Approved without modification  
☒ Approved with modification - Supplemental Information Form Attached - ☒ Yes ☐ No

Test concentrations 50 & 200 ppm as Bromine

APPROVAL SIGNATURES

SPONSOR:

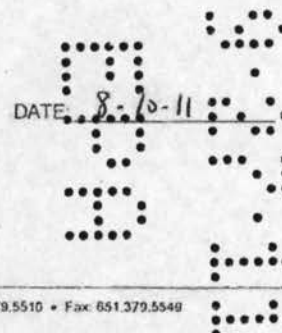
NAME: Mr. Eric Iimatta TITLE: R & D Advisor  
SIGNATURE: [Signature] DATE: 7/22/11  
PHONE: (225) 359-2972 FAX: (225) 768-5990 EMAIL: eric.iimatta@albemarle.com

For confidentiality purposes, study information will be released only to the sponsor/representative signing the protocol (above) unless other individuals are specifically authorized in writing to receive study information.

Other individuals authorized to receive information regarding this study: ☐ See Attached

ATS Labs:

NAME: Anne Skemp  
Study Director  
SIGNATURE: [Signature]  
Study Director



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Note: AOAC Fluid Glycollate Broth with 0.5% Na Thiosulfate was used in a previous study with Salmonella as the test organism. I don't know if that is the appropriate subculture broth for the test organisms in this study but thiosulfate is the appropriate neutralizing agent.

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#### Stock Solution Preparation:

In a flask add in the ratio of approximately 1 g of DBDMH powder to approximately 500 ml of deionized water. Stir magnetically for approximately 30 minutes. Filter off the insoluble material. Please analyze within 1 hour of sample preparation.

#### Analytical Procedure

- (1) Pipette 50.0 ml of stock solution of DBDMH into a 250 ml Erlenmeyer flask containing approximately 50 ml of de-ionized water.
- (2) Add 1 g KI crystals
- (3) Add 5 ml glacial acetic acid or sufficient to bring to pH below 4.
- (4) Titrate the iodine which is liberated with 0.1 N sodium thiosulfate to discharge the red/yellow color.
- (5) Towards the end point, the solution will have a pale straw color. At this point, add 1 ml of starch indicator. The solution will turn blue/black.
- (6) Continue the titration by slowly adding the sodium thiosulfate, one drop at a time, to discharge to the blue/black coloration to yield a colorless solution. Record the volume of titrant, and report the result to the nearest 0.1 ml. This is volume A.
- (7) Perform a reagent blank, by repeating the titration in the absence of DBDMH stock solution. This is volume B. Volume B may be zero if the reagents do not elicit a color response in the presence KI.

#### Calculation

$$\text{mg/L bromine equivalent} = \frac{(A-B) \times N \times 79.9 \times 1000}{SV}$$

Where: A is ml titration for sample

B is ml titration for reagent blank

N is normality of sodium thiosulfate, which in this case is 0.1 N

SV is sample volume taken for titration which in this case is 50.0 ml

Per Sporn request, each test substance  
sample will be titrated in duplicate.  
in 8-10-11



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Using this procedure, an equivalent bromine concentrate of around 1100-1250 mg/L will be prepared.

This stock solution should be diluted with chlorine demand free sterile water to achieve the desired test concentrations. The test concentration materials should be used with 3 hours of preparation.

Any questions I can be reached at 225 359-2972 (Eric Liimatta)

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